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14. ABSTRACT Cancer associated fibroblasts (CAFs) are a major stromal cell type in breast tumors and produce several inflammatory cytokines required for breast cancer progression. Stromal fibroblasts modulate breast cancer cell behavior through diverse mechanisms, including the synthesis of pro-inflammatory cytokines, as well as remodeling of the extracellular matrix (ECM). Overall, the cell biological mechanisms controlling the pro-tumor functions of stromal fibroblasts, including CAFs, remain poorly understood. This proposal seeks to understand, how autophagy, a tightly regulated lysosomal degradation process, modulates the tumor-promoting properties of stromal fibroblasts. During the first year of this proposal, we have optimized the conditions to isolate both normal mammary gland fibroblasts as well as tumor derived CAFs. Furthermore, we have successfully these fibroblasts into the mammary fat pad of syngeneic recipient mice in combination with polyoma middle T (PyMT) breast cancer cells. Our pilot studies indicate that PyMT tumor growth is impaired in the presence of autophagy deficient, but not autophagy-competent fibroblasts, providing initial support for the hypothesis that autophagy in stromal fibroblasts is essential for breast cancer progression. In addition, we have uncovered that autophagy-deficient fibroblasts exhibit reduced secretion of multiple pro-inflammatory cytokines in vitro as well as exhibit reduced contractility in type I collagen in vitro. In the upcoming year, we will validate these results and determine whether and how changes in cytokine secretion and ECM remodeling impact breast cancer progression in vivo.					
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I. INTRODUCTION:

Tumor cells coexist with immune cells, fibroblasts, and blood vessels, which is collectively termed the tumor stroma. This complex, dynamic cellular network creates a microenvironment that supports primary tumor cell growth, as well as invasion and metastasis. Cancer associated fibroblasts (CAFs) are the most abundant stromal cell type in breast tumors and produce several inflammatory cytokines required for breast cancer progression[1-3] [4]. Fibroblasts modulate breast cancer cell behavior through diverse mechanisms, including the synthesis of growth and angiogenic factors, as well as ECM components and proteases. Indeed, many tumors exhibit striking histological evidence of fibroblast proliferation and activation, termed desmoplasia[5,6]. Desmoplasia correlates with poor prognosis, yet very little is known about the molecular mechanisms required to sustain the desmoplastic response[5,6] Overall, the cell biological mechanisms controlling the pro-tumor functions of stromal fibroblasts, including CAFs, remain poorly understood. Our overall **hypothesis** is that autophagy, a tightly regulated lysosomal degradation process, in stromal fibroblasts is essential for breast cancer progression. This proposal seeks to understand: (1) how autophagy inhibition in stromal fibroblasts modulates their tumor promoting properties, and (2) how autophagy inhibition in fibroblasts impacts their secretion of specific factors that govern aggressive behavior of breast cancer cells. For our studies, we are utilizing the Polyoma Middle T (PyMT) breast cancer model to evaluate how the specific lack of autophagy in fibroblasts impacts mammary cancer growth and progression in an immunocompetent model.

During the first year of this proposal, we have obtained strong support for our hypothesis. We have focused on the following goals and tasks: (1) We have successfully optimized the conditions necessary to isolate both normal mammary gland fibroblasts as well as PyMT-tumor derived cancer associated fibroblasts. (2) We have co-transplanted these fibroblasts into the mammary fat pad of syngeneic recipient mice with breast cancer cells; our pilot studies indicate that PyMT tumor growth is severely impaired in the presence of autophagy deficient, but not autophagy-competent fibroblasts. (3) We have uncovered that autophagy-deficient cells exhibit reduced production of multiple pro-inflammatory cytokines and pro-invasive secreted factors. During the second year, we will build upon these results to investigate how autophagy in stromal fibroblasts promotes the breast cancer growth and progression.

II. RESEARCH ACCOMPLISHMENTS BODY:

Task 1. Determine the effects on genetic ATG deletion in stromal fibroblasts on mammary tumor progression.

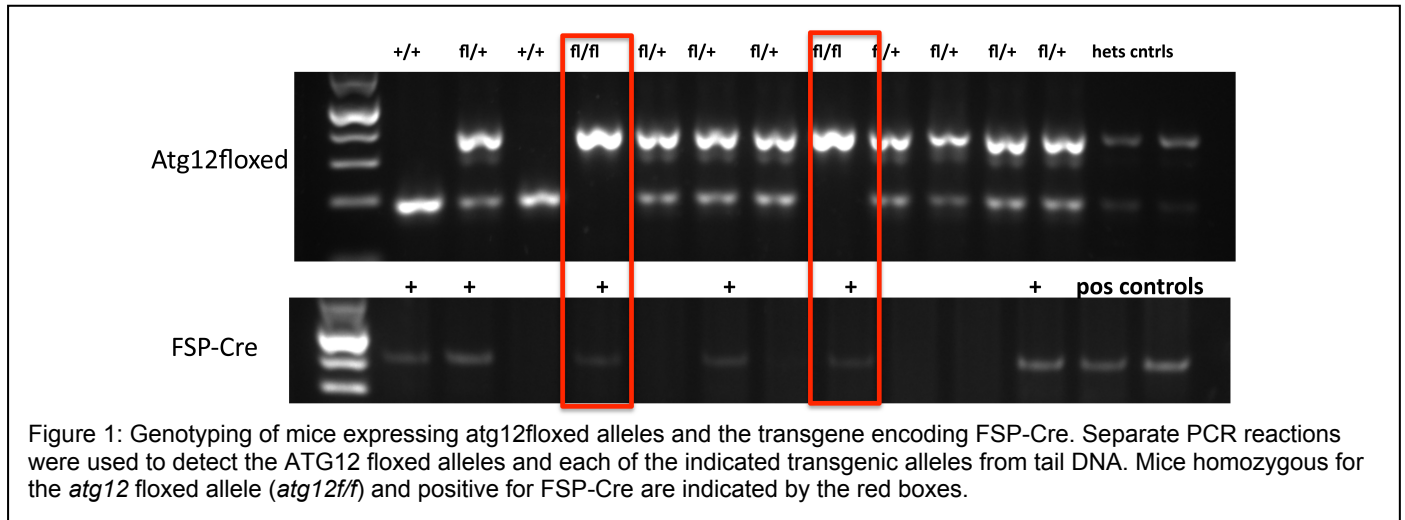
a. Obtain regulatory approval (ACURO) for studies using transgenic mice (Months 1-3).

ACURO approval for BC113181 was successfully obtained.

b. Intercross transgenic mice expressing conditional null alleles (floxed) of autophagy regulators (e.g., *atg5* and *atg12*) with FSP-Cre transgenic mice (Months 4-9).

The fibroblast specific protein (FSP) promoter (FSP-Cre). The FSP promoter has been shown to effectively delete various genes in fibroblasts [5,7]. We intercrossed of FSP-Cre mice with the ATG12 conditional mice (*atg12^{f/f}*) and ATG5 conditional mice (*atg5^{f/f}*). We successfully obtained mice carrying both the FSP-Cre transgene as well as homozygous for the *atg12^{f/f}* allele; genotyping data in Figure 1 shows two animals that co-express these alleles. These mice are viable and fertile, which has made them suitable for the intercrosses in subtask 1c. Because our mouse colony of *atg5^{f/f}* mice has unexpectedly bred more slowly over the past year, the generation of these transgenic strains is still in progress. We do not anticipate that this delay will impact our results, because we will use the ATG5 deletion strain to validate results in the ATG12 model; hence, we anticipate that the we will need lower numbers of these animals for certain key experiments. Moreover, our results described below in subtasks 1f and 2a suggests that we will be able to

use our transplantation models to validate results in both ATG12 and ATG5 deficient fibroblasts; hence, we will be able to confirm that our phenotypes are due to a broader defect in stromal autophagy, as opposed to a phenotype attributable to an individual ATG, the ultimate goal of this project.



- c. **Generate transgenic mice expressing MMTV-PyMT (Polyoma Middle T Antigen), FSP (Fibroblast specific protein)-Cre, and conditional null alleles (floxed) of autophagy regulators (e.g., *atg5* and *atg12*) in a pure genetic (C57B/6) background (Months 4-12).**

The intercross of FSP-Cre *atg12^{fl/fl}* mice with PyMT mice has been initiated. One potential caveat that has arisen is that fibroblast specific protein (FSP) may be expressed at low levels in late stage PyMT tumor cells exhibiting mesenchymal differentiation, as shown in Figure 2. Thus, the FSP-Cre driver may delete ATGs in late stage PyMT tumor cells as well as stromal fibroblast cells, which may confound the interpretation of our results. Although these results do not obviate the use of this model, we have placed higher priority on the use of co-transplantation strategies in subtask 1f, because in such models we are more confident of a fibroblast-specific deletion. We initiated these studies ahead of schedule and already obtained interesting results in support of our hypothesis, as described below in subtask 1e. In addition, we have discovered that Platelet Derived Growth Factor Receptor B (PDGFRB) is highly expressed in the intervening fibroblastic stroma but not in PyMT tumor cells (Figure 3). As a result, the use of a Cre recombinase driven by the PDGFRB promoter may be a superior strategy to achieve fibroblast-specific deletion of autophagy; hence, we have initiated the interbreeding of *atg12^{fl/fl}* with PDGFRB-Cre to deal with this critical issue.

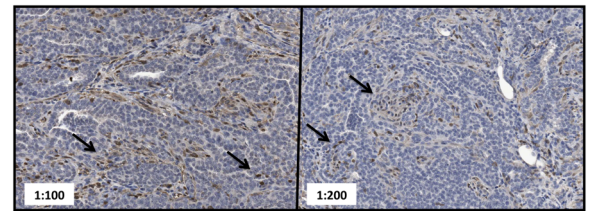


Figure 2: Evidence of FSP expression in PyMT tumor cells. Advanced PyMT tumors (4 months) were analyzed by immunohistochemistry using antibodies against FSP at the indicated dilutions. Black arrows indicate PyMT tumor cells that are FSP positive.

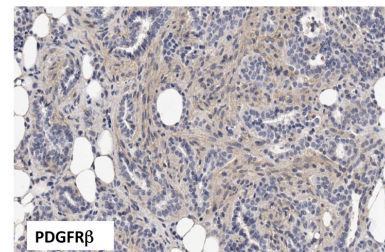


Figure 3: PDGFR β expressed in the intervening stroma of PyMT tumors. PyMT tumors were analyzed by IHC using antibodies against PDGFR β .

- d. **Establish routine isolation and short-term culture conditions for primary murine fibroblasts and CAFs isolated from ATG conditional mice (*atg5^{fl/fl}* and *atg12^{fl/fl}*) (Months 4-8).**

We have developed optimized the reagents and the protocols for the isolation of primary fibroblasts and CAFs from the mammary glands of ATG conditional mice (*atg5f/f* and *atg12f/f*). The details of our standard procedures are as follows:

Protocol 1: Primary mammary fibroblast isolation

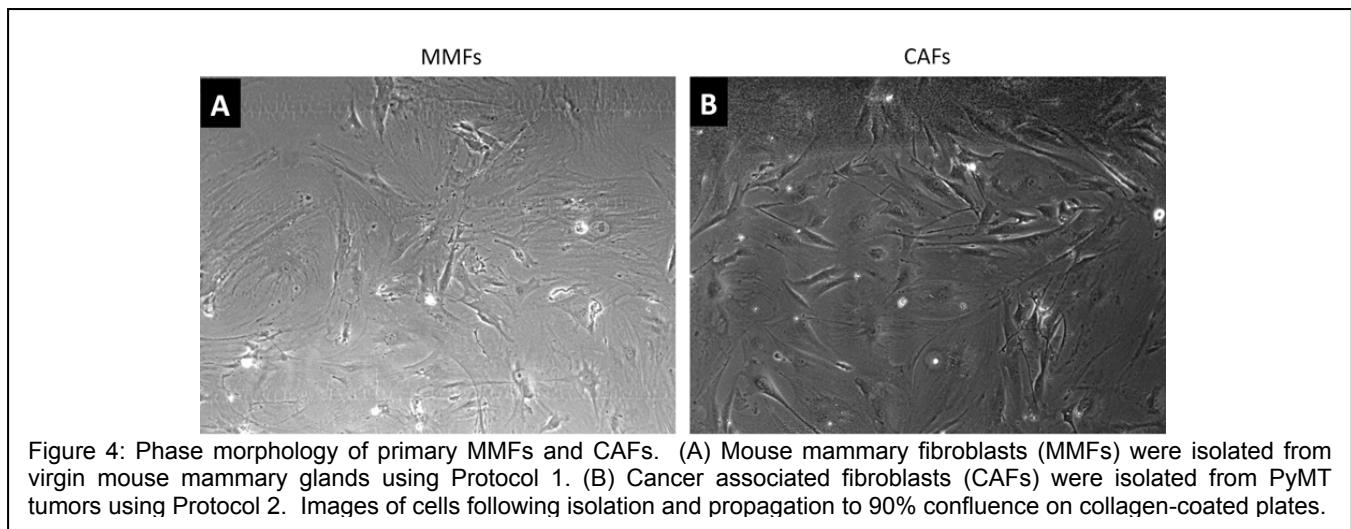
Reagents:

DMEM + 10% Calf Serum (NOT heat inactivated) + 1% Penicillin/Streptomycin (*MMF media*)
 50 mg/ml gentamicin
 Collagenase (Sigma #C5138)
 Hyaluronidase (Sigma #H3506)
 PBS
 Collagen coated tissue culture plates

Digestion Solution to be made fresh right before use:

(prepare 5 mls digestion solution per MFP)
 1.5 mg/ml Collagenase (from 100X stock solution)
 125 U/ml Hyaluronidase (from 100X stock solution)
 MMF media + 50 µg/ml gentamicin
 Filtered through a 0.22 µm filter

1. Euthanize mice and harvest 3rd and 4th mammary glands (also 5th if you want) using aseptic technique in the necropsy room.
2. Leave mammary glands in PBS on ice while preparing digestion solution (see above)
3. Mince tissue with a sterile scalpel in hood.
4. Add digestion solution and minced tissue to autoclaved 50 ml jars with magnetic stir bars, using ratio above.
5. Incubate in water bath shaking at 37 °C for 30 min. Digestion is complete when media is cloudy and few tissue chunks remain. A large amount of tissue chunks can be dissociated using an 18G needle/syringe if necessary.
6. Inactivate collagenase by adding 1 uM EDTA.
7. Spin down 1000 rpm 5 min.
8. Resuspend pellet in MMF media + 50 ug/ml gentamicin and plate on a p60 (cells grow better when plated more densely at the start) on collagen coated plates. The morphology of fibroblasts post-propagation in culture is shown in Figure 4A.



Protocol 2: Isolation of cancer-associated fibroblasts.**Reagents:**

DMEM + 10% Calf Serum (NOT heat inactivated) + 1% Penicillin/Streptomycin (*MMF media*)

PBS + 0.5% Bovine Serum Albumin (*FACS Buffer I*)

PBS + 5% Calf Serum (*FACS Buffer II*)

50 mg/ml gentamicin

Collagenase (Sigma #C5138)

Hyaluronidase (Sigma #H3506)

Red Blood Cell Lysis Buffer (Sigma #R7757)

PDGFR α antibody, PE conjugated (eBiosciences #12-1401)

F4/80 antibody, APC conjugated (eBiosciences #17-4801)

PBS

Collagen coated plates.

Digestion Solution to be made fresh right before use:

(prepare 20 mls digestion solution per 5g of tumor tissue)

1.5 mg/ml Collagenase (from 100X stock solution)

125 U/ml Hyaluronidase (from 100X stock solution)

MMF media + 50 μ g/ml gentamicin

Filtered through a 0.22 μ m filter

1. Euthanize mice and harvest 4th and 5th mammary glands using aseptic technique in necropsy room.
2. Weigh tumor burdened mammary glands
3. Leave mammary glands in PBS on ice while preparing digestion solution (see above).
4. Mince tissue with a sterile scalpel in hood.
5. Add digestion solution and minced tissue to autoclaved 50 ml jars with magnetic stir bars, using ratio above.
6. Incubate in waterbath shaking at 37 °C for 30 min. Digestion is complete when media is cloudy and few tissue chunks remain.
7. Dilute digestion medium at least 2X the volume with MMF media.
8. Inactivate collagenase by adding 1 uM EDTA.
9. Remove large, undigested tissue chunks with a 70 μ m filter. Wash filter with media before discarding.
10. Spin down 1000 rpm 5 min.
11. Perform an RBC lysis: Incubate the pellet for 1 min at RT in 1ml of RBC Lysis Buffer.
12. Immediately add 20 mls of PBS
13. Spin down 1140 rpm 7 min
14. Repeat steps 12 and 13.
15. Resuspend pellet in MMF media + 50 ug/ml gentamicin.
16. Count cells. During any downtime, keep cells on ice.
17. Spin down 1000 rpm 5min.
18. Resuspend cells in FACS Buffer II at an appropriate cell concentration for staining. Depending on cell number, this will range from 10⁷ to 10⁸ cells/ml. Keep cells on ice.
19. Allocate cell suspension to 5ml polypropylene FACS tubes and prepare essential staining control tubes (keeping cells on ice); ~500 ul per tube or less for total volume.
 - a. 100 ul of unstained cell suspension
 - b. 100 ul of isotype cell suspension if we have it in lab (rat IgG2a for both)
 - c. 100 ul of PDGFR α -PE alone
 - d. 100 ul of F4/80-APC alone
20. Add antibodies:

- a. 1:50 dilution of PDGFR α -PE
- b. 1:50 dilution of F4/80-APC
21. Incubate on ice for 30 min in the dark.
22. Wash cells with at least 1 ml FACS Buffer II per tube.
23. Spin down 1000 rpm 5 min
24. Resuspend pellet in 250 μ l FACS Buffer II.
25. Keep cells on ice and head to FACS sorter. Remember to bring 15 ml collection tubes with at least 5 ml MMF media to retrieve sorted cells, as well as extra FACS Buffer II for dilution.
26. Spin down sorted cells 1000 rpm 5 min.
27. Resuspend in MMF media and plate on a p60 (cells grow better when plated more densely at the start) on collagen-coated plates. The morphology of CAFs post-propagation in culture is shown in Figure 4B.

e. Optimize protocols for the stable *ex vivo* transduction of Cre recombinase and fluorescent, luminescent, and/or drug selection marker proteins into stromal fibroblasts (Months 4-12).

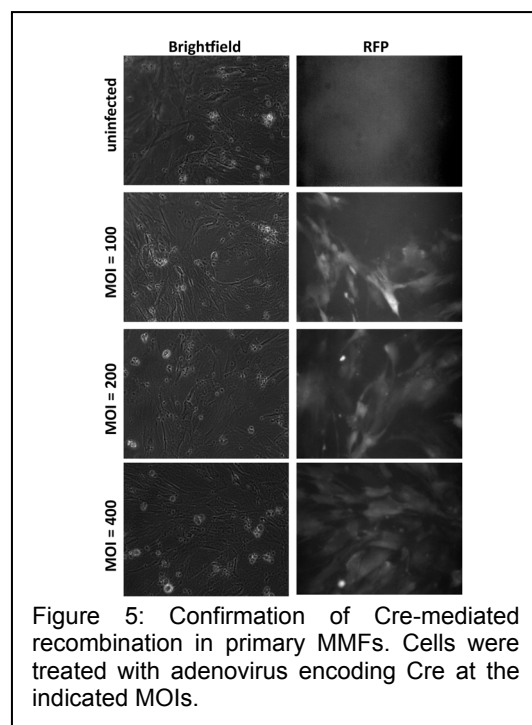


Figure 5: Confirmation of Cre-mediated recombination in primary MMFs. Cells were treated with adenovirus encoding Cre at the indicated MOIs.

We have optimized the delivery of adenoviral Cre recombinase. Our cells include an LSL-RFP reporter to confirm Cre-mediated recombination; they also allow us follow cells both in vitro and in vivo for tracking purposes, obviating the need for exogenous marker introduction as originally proposed.

1. 1×10^6 fibroblasts or CAFs are plated in MMF media on collagen-coated dishes on the night prior.
2. Adenovirus containing Cre recombinase (viral particles obtained from Developmental Studies Hybridoma, University of Iowa) is added to wells; the amount to virus is determined empirically for each batch; we have found that an MOI of 100-400 for adeno-Cre has been found to provide 80-95% infection efficiency of fibroblast cultures, based on (See Figure 5). When desired, parallel cultures can also be treated with adenovirus expressing empty vector (EV) as a control.
3. Cells are incubated with adenoviral particles for 1 hr at 37°C, 5% CO₂ in a tissue culture incubator, upon which 500 μ l growth medium is added, and the culture is incubated for an additional 48 hr.

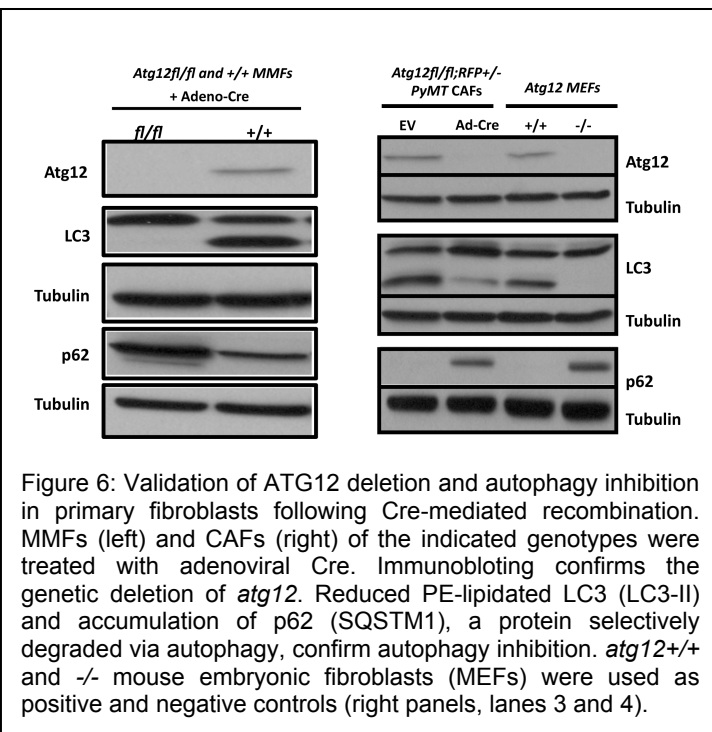


Figure 6: Validation of ATG12 deletion and autophagy inhibition in primary fibroblasts following Cre-mediated recombination. MMFs (left) and CAFs (right) of the indicated genotypes were treated with adenoviral Cre. Immunoblotting confirms the genetic deletion of *atg12*. Reduced PE-lipidated LC3 (LC3-II) and accumulation of p62 (SQSTM1), a protein selectively degraded via autophagy, confirm autophagy inhibition. *atg12*^{+/+} and *-/-* mouse embryonic fibroblasts (MEFs) were used as positive and negative controls (right panels, lanes 3 and 4).

4. Genetic deletion is confirmed in vitro prior to transplantation studies in mice or in vitro functional studies. This is accomplished via immunoblotting for ATG12 or ATG5. Autophagy inhibition is

confirmed by immunoblotting for reduction of the PE-lipidated isoform of LC3-II and the accumulation of p62/SQSTM1, a protein that is selectively degraded via autophagy, as shown in Figure 6.

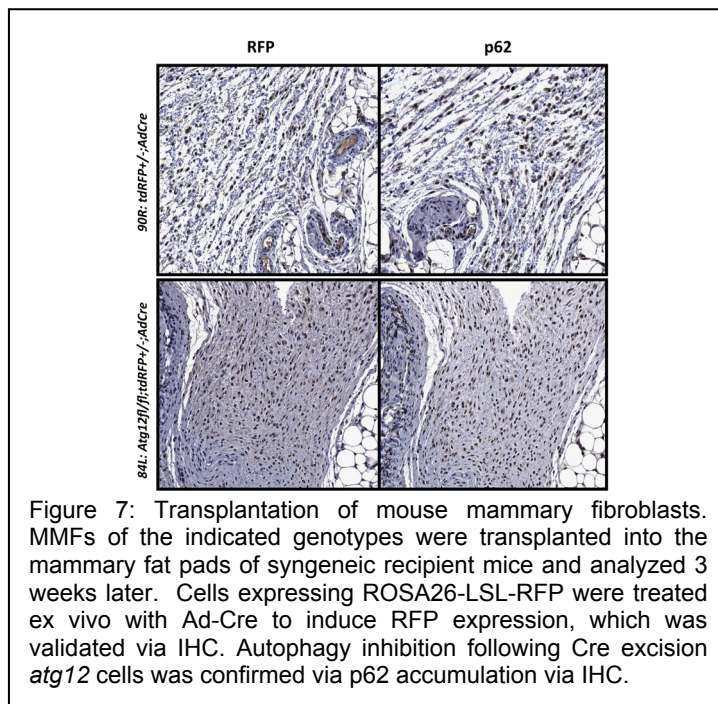
- f. Transplant fibroblasts from subtask 1e and PyMT mammary cancer cells into cleared mammary fat pads of host recipient animals (either NOD/SCID for pilot studies or syngeneic C57B/6 hosts for experiments). For this subtask, we anticipate that fifteen (15) donor mice will be utilized for fibroblast and PyMT epithelial isolation, and thirty (30) host recipient animals per cohort will be utilized for fat pad transplantation (Months 9-15).**

We have successfully transplanted fibroblasts into the cleared pad of mouse mammary glands of both NOD/SCID animals as well as C57B/6 syngeneic hosts. As shown in Figure 7, control and ATG12 deleted fibroblasts generated from subtask 1d-e were transplanted into C57B/6 animals; at 3 weeks post-transplantation, mice were euthanized and immunohistochemical analysis confirmed the presence of viable fibroblasts with evidence for RFP expression as well as increased p62 expression, corroborating both Cre mediated deletion of ATG12 as well as autophagy inhibition. Overall, these results confirm that our ex vivo manipulations do not impact the subsequent incorporation of these cells, both autophagy competent and deficient into an immunocompetent host.

We have pursued two strategies for the co-transplantation of PyMT cells with stromal fibroblasts. First, following the initial transplantation of stromal fibroblasts, we introduce PyMT tumor cells 3 weeks later into the primed mammary fat pad. This priming strategy is based on an established technique by the Charlotte Kuperwasser laboratory (Tufts University) that has been used to interrogate the role of human CAFs in tumor progression [8]. Second, we have co-injected fibroblasts from subtasks 1d-e with PyMT breast tumor cells (at a ratio of 3:1 fibroblasts to PyMT tumor cells) into the cleared fat pads of syngeneic female recipient mice. We have already obtained results from three pilot experiments (4-6 animals per cohort), which are described in subtask 1g below. Additional experiments to increase the number of animals per cohort have already been initiated or will be started during year 2; through these studies, we will be able to evaluate multiple time points for the parameters described in subtask 1g.

- g. Determine the latency period to the onset of primary tumor formation and metastasis for recipient mice generated in subtask 1f. At selected time points, ten (10) mice from each experimental cohort will be functionally evaluated for altered characteristics of histopathologic progression from primary to metastatic disease (mammary morphogenesis, epithelial cell proliferation/cell death, overt tumor formation, tumor burden, tumor type, and development/latency to metastases) (Months 12-24).**

We have conducted pilot studies to determine how the specific ablation of autophagy in fibroblasts impacts primary tumor formation in mice. In the first pilot experiment, cleared mammary fat pads were primed with 2.5×10^5 fibroblasts, either autophagy competent or deficient (due to ATG12 deletion), followed by the injection of 75,000 PyMT autophagy competent tumor cells 3 weeks later. In the second pilot experiment,



priming was conducted using 5.0×10^5 fibroblasts, followed by the introduction of 150,000 PyMT cells 3 weeks later. In both experiments, 5 animals per cohort were analyzed for onset and growth of primary tumors. Although the latency to tumor formation differs between the two experiments (for control cells, 50 days to tumor onset in pilot 1 and 35 days in pilot 2), both experiments demonstrate a delay in tumor formation as well a reduction in overall tumor growth when animals are primed with ATG12 deficient fibroblasts versus autophagy competent controls (Figure 8). In parallel, we performed a co-mixing experiment; into the cleared fat pads of syngeneic female recipient mice (6 animals per cohort), we co-injected 225,000 fibroblasts, either autophagy competent (*atg12*^{+/+}, Cre-treated) or autophagy deficient (*atg12*^{f/f}, Cre treated), in combination with 75,00 PyMT cells, maintaining a ratio of 3:1 fibroblasts to PyMT tumor cells. Once again, the onset and overall growth of primary PyMT tumor was decreased in animals injected with ATG12 null fibroblasts compared to those injected wild type fibroblasts (Figure 9).

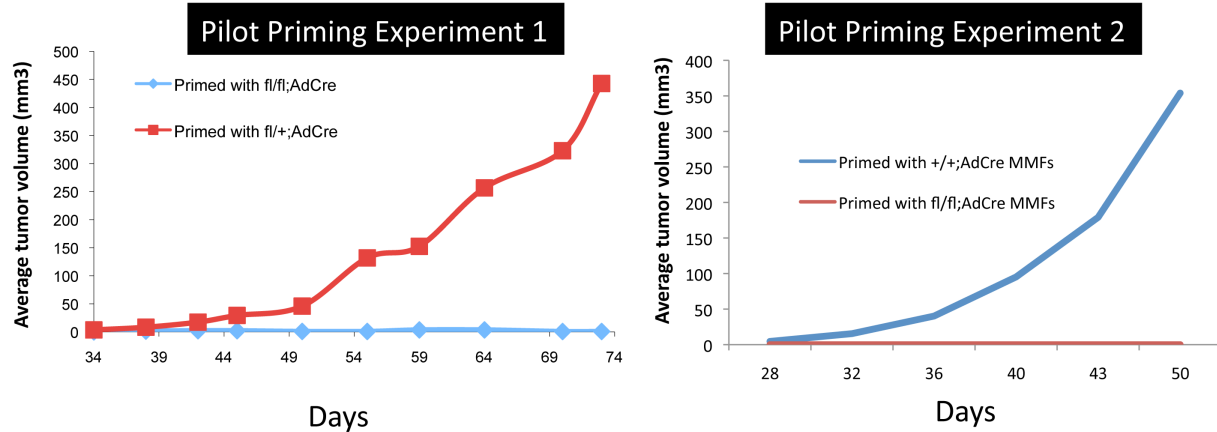


Figure 8: Priming with autophagy deficient fibroblasts delays onset and impedes the growth of PyMT tumors. In two pilot experiments, autophagy deficient (*atg12*^{f/f}) and competent (*f*^{+/+} or *+/+*) fibroblasts were used to prime mammary fat pads of syngeneic C57B/6 mice. PyMT tumor cells were injected into the primed fat pad three weeks later and average tumor volume was monitored over the indicated time course. For each experiment, 5 animals per cohort were analyzed. (Note that red vs. blue color coding of the line graphs is reversed between experiments.)

Overall, these experiments provide strong support for our hypothesis that autophagy deficiency in stromal fibroblasts can short circuit tumor progression by impeding the growth of autophagy competent tumor cells. We will now conduct the experiments using a larger number of mice per cohort during the upcoming year. For both of these strategies, we will evaluate two time points for altered characteristics of histopathologic progression from primary to metastatic disease. Based on our pilot experiments, our plan for the priming studies is to utilize a standard protocol of 5.0×10^5 fibroblasts, followed by the introduction of 150,000 PyMT cells. In addition to these studies with stromal mammary gland fibroblasts, we will pursue experiments using CAFs derived from PyMT tumors.

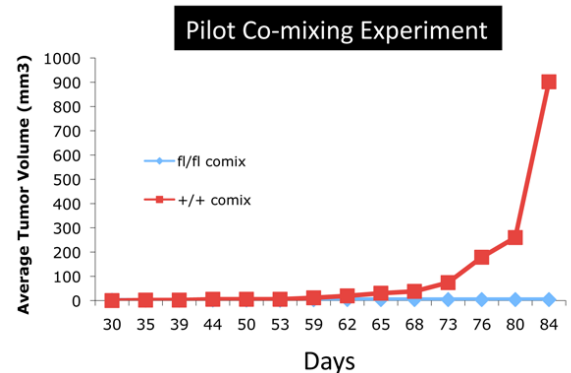


Figure 9: Co-mixing with autophagy-deficient fibroblasts delays onset and impedes the growth of PyMT tumors. Cleared mammary fat pads were co-transplanted with PyMT tumor cells and autophagy deficient (*atg12*^{f/f}+Cre) and competent (*+/+*+Cre) fibroblasts. average tumor volume was monitored over the indicated time course. n=6 animals per cohort.

Task 2. Determine how ATG deletion in stromal fibroblasts influences breast cancer cell fate and behavior.

- a. **Perform cytokine arrays on conditioned media produced from ATG deficient fibroblasts, and validate results using ELISA. Initially, we will use immortalized *atg5*^{-/-} and *atg12*^{-/-} mouse embryonic fibroblasts; subsequently, we will corroborate important results using primary cancer-associated stromal fibroblasts (CAFs) generated from subtask 1d (Months 1-12; Revised timeline Months 1-24).**

Using the protocols described in subtask 1d, we isolated mouse mammary glands from *atg5*^{f/f} and *atg12*^{f/f} mice; thereafter, the primary cultures were treated with adenoviral Cre according to the protocol described in subtask 1e. Although we originally planned to utilize immortalized MEFs, we were fortunate in our attempts to generate primary murine fibroblasts derived from the mouse mammary glands, which comprised a far superior source of fibroblasts for the studies in this subtask. Thus, we performed cytokine arrays from the conditioned media produced from primary *atg5*^{-/-} and *atg12*^{-/-} stromal fibroblasts derived from mouse mammary glands. The fibroblast cultures were grown to 90% confluence upon which the MMF media was replaced with serum free DMEM for 12 hours. Subsequently the media was collected and subject to analysis via cytokine semi-quantitative antibody arrays (RayBiotech “C-series”). These arrays utilize the sandwich immunoassay principle, where a panel of capture antibodies is printed on a nitrocellulose membrane solid support. The array membranes were incubated with the conditioned media from control (empty vector, EV) and ATG deficient (Cre) fibroblasts, processed via enhanced chemiluminescence, upon which signals were imaged on X-ray film, and subject to densitometry data collection and the calculation of fold-changes for each detected protein. Using this method, we compared the production of 164 mouse cytokines in ATG12 and ATG5 deficient mammary fibroblasts (Cre treated) as well as their respective autophagy competent (EV) controls; the normalized fold changes for 17 representative cytokines from these arrays are shown in Figure 10.

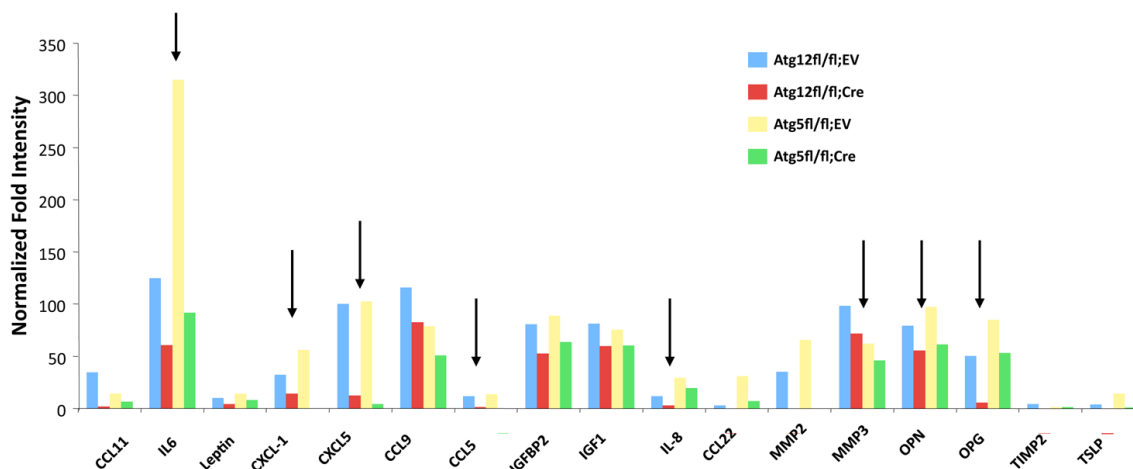


Figure 10: In vitro cytokine secretion by autophagy deficient fibroblasts. Conditioned media (CM) produced from the indicated cell types was analyzed for the production of 164 mouse cytokines on a semi-quantitative antibody array (Ray Biotech). Normalized fold intensity of 17 representative molecules is shown; arrows indicate cytokines that exhibit reduced production in both ATG12 and ATG5 deficient fibroblast (Cre) in comparison to controls (EV).

To prioritize candidates for further analysis, we identified molecules that were reduced upon genetic deletion of either ATG5 or ATG12, because changes in these factors were more likely to result from a general defect in autophagy rather than the specific deletion of an individual ATG. Based on the analysis of two biological replicates, eight pro-inflammatory cytokines, chemokines, and pro-invasive factors met this criteria and were secreted at reduced levels in both ATG5 and ATG12 deficient fibroblasts (Figure 10, arrows) in comparison to their respective controls (EV); they include: IL6, IL5, CXCL1, CXCL5, CCL5, IL8,

MMP3, OPN, and OPG. We have begun to validate results via ELISA; specifically, we have confirmed that IL6 secretion is reduced in autophagy deficient fibroblasts (Figure 11). These findings are consistent with an emerging role for autophagy in secretion. Though traditionally viewed as an autodigestive process, autophagy also facilitates biosynthesis and secretion, both functions that are being increasingly appreciated. Indeed, early evidence indicates a genetic requirement for ATGs during 1) unconventional secretion of proteins lacking an N-terminal ER signal sequence [9-11]; 2) the efficient egress of secretory lysosomes[12]; and 3) conventional secretion of growth factors [13,14]. Moreover, autophagy plays an important role in promoting inflammatory cytokine secretion during oncogene-induced senescence (OIS); it supports the production of interleukin-6 (IL6) and interleukin-8 (IL8), two cytokines associated with the “senescence associated secretory phenotype” (SASP) that are secreted via conventional pathways[15]. Hence, we will continue to corroborate these results in both normal mammary fibroblasts and CAFs over the upcoming year.

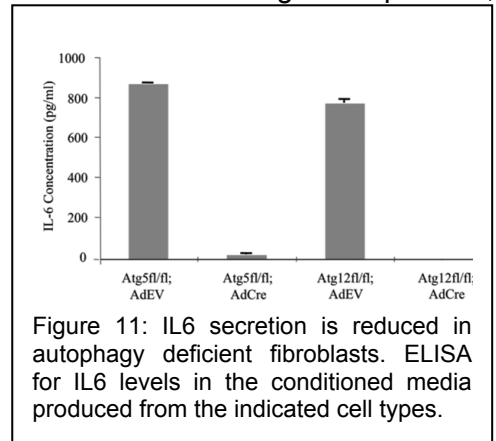


Figure 11: IL6 secretion is reduced in autophagy deficient fibroblasts. ELISA for IL6 levels in the conditioned media produced from the indicated cell types.

- b. Establish three-dimensional culture systems using ATG deficient fibroblasts and wild type controls. For pilot experiments, we will utilize immortalized fibroblasts, and subsequently, interrogate primary CAFs generated from subtask 1d. Important parameters that we will evaluate include the effects of fibroblast migration/invasion, cytokine secretion in 3D, proliferation/cell death, and fibroblast-mediated gel retraction (Months 1-12, Revised timeline Months 1-24).**

To more faithfully recapitulate the microenvironment of stromal fibroblasts, we cultured these cells within a collagen I-based matrix for 3D culture studies. We embedded primary fibroblasts ($1-5 \times 10^5$) into activated rat-derived collagen I to create a fibroblast-containing collagen gel. During year 1, we successfully optimized a collagen gel contraction assay, a commonly used method to study cell-mediated reorganization of the surrounding extracellular matrix [16]. As shown in Figure 12, the degree of fibroblast-mediated gel retraction was calculated by measuring the area of the disc over a time course of 6-24h following the embedment of cells into the collagen I gel. Interestingly, our preliminary studies indicate that ATG12 $-/-$ primary MEFs have a reduced ability to contract type I collagen compared to their WT counterparts, even during the initial stages of contraction following 6h of treatment with transforming growth factor beta ($TGF\beta$), a fibrogenic hormone (Figure 12). These results suggest autophagy competency in fibroblasts enables collagen remodeling in vitro and support our prediction that autophagy promotes the desmoplastic response associated with a poor prognosis in breast cancer [3]. We will build on these exciting in vitro results during year 2 and further investigate the role of fibroblasts on ECM remodeling and desmoplasia in vivo as part of subtask 1g as well as interrogate the collagen retraction rates of autophagy-deficient CAFs in vitro. We are continuing to optimizing the methods

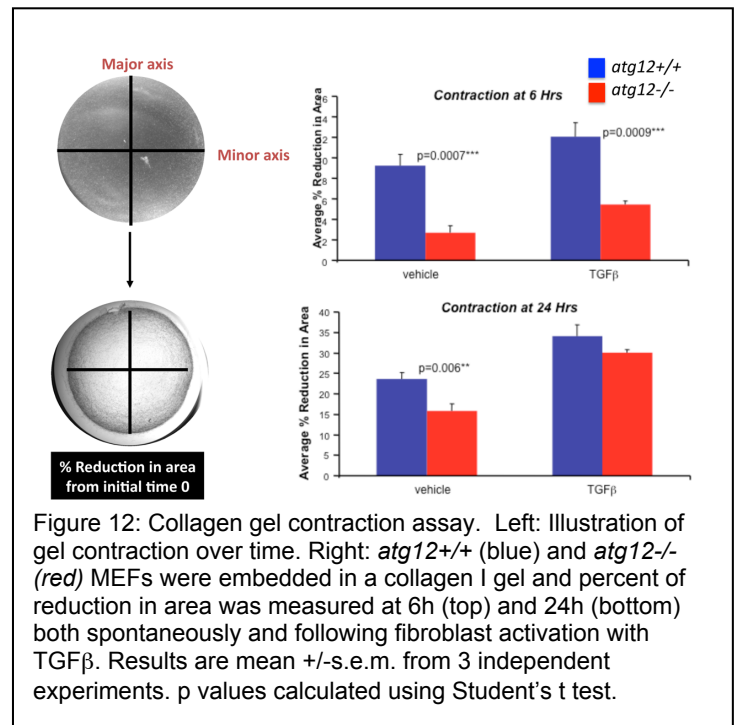


Figure 12: Collagen gel contraction assay. Left: Illustration of gel contraction over time. Right: *atg12*^{+/+} (blue) and *atg12*^{-/-} (red) MEFs were embedded in a collagen I gel and percent of reduction in area was measured at 6h (top) and 24h (bottom) both spontaneously and following fibroblast activation with $TGF\beta$. Results are mean \pm s.e.m. from 3 independent experiments. p values calculated using Student's t test.

to quantify the fibroblast proliferation/cell death, migration and cytokine secretion in these 3D gels and plan to complete these remaining studies during the upcoming year.

- c. **Establish and analyze co-culture assays using ATG deficient fibroblasts with PyMT-derived tumor cells. Important parameters that we will evaluate in co-culture include PyMT tumor cell proliferation/cell death, secretion of cytokines identified in subtask 2a, and ECM invasion and proteolysis (Months 12-24).**

These studies are planned for the upcoming year.

- d. **Obtain and analyze gene expression profiles from primary fibroblasts and CAFs isolated from ATG conditional mice (*atg5f/f* and *atg12f/f*). Priority will be placed on quantifying the expression of candidate cytokine identified in subtask 1a (Months 12-24).**

These studies are planned for the upcoming year. Because of positive results from the cytokine array, we will initially conduct quantitative PCR studies to measure the mRNA expression of specific cytokine candidates in subtask 2a prior to conducting a broader unbiased analysis of gene expression.

III. KEY RESEARCH ACCOMPLISHMENTS:

- 1) We have successfully intercrossed FSP-Cre with *atg12f/f* to achieve fibroblast-specific deletion and obtained viable, fertile animals. Intercrossing of FSP-Cre with *atg5f/f* is underway.
- 2) We initiated the intercross to create compound transgenic mice expressing MMTV-PyMT (Polyoma Middle T Antigen), FSP (Fibroblast specific protein)-Cre, and conditional null alleles (floxed) of autophagy regulators.
- 3) We have successfully optimized the isolation of primary mammary fibroblasts and CAFs from mice and the adenoviral delivery of Cre recombinase into *atgf/f* cells. We have also confirmed the genetic deletion of ATG5/ATG12 as well as the inhibition of autophagy in these fibroblast populations.
- 4) We have transplanted fibroblasts and PyMT tumor cells into the fat pad of recipient mice using two different strategies-priming and co-mixing.
- 5) Our pilot experiments indicate that autophagy deficient fibroblasts impede the growth of primary PyMT tumors, providing initial evidence in support of our hypothesis. We will validate this result in the upcoming year.
- 6) We have uncovered that autophagy-deficient fibroblasts exhibit reduced secretion of multiple pro-inflammatory cytokines in vitro, including IL-6, CXCL-1, and CXCL-5.
- 7) We have uncovered that autophagy-deficient fibroblasts exhibit reduced contractility in type I collagen in vitro, broaching the idea that reduced tension in vivo may contribute to reduced primary tumor growth.

IV. REPORTABLE OUTCOMES:

Publications (Months 1-12):

None.

Presentations (Months 1-12):

International and National Conferences:

- 2012 Invited Speaker, 6th International Symposium on Autophagy, Okinawa, Japan
- 2012 Invited Speaker, American Society for Cell Biology National Meeting, Minisymposium on Cancer Cell Biology, San Francisco, CA
- 2013 Plenary Speaker, Applied Pharmaceutical Toxicology Meeting, Genentech Inc., South San Francisco, CA

Invited Lectures and Seminars:

- 2012 Invited Speaker, 1st Annual Helen Diller Family Cancer Center Annual Retreat, Santa Cruz, CA
- 2012 Invited Seminar, Department of Physiology, University of Texas Health Science Center, San Antonio, TX
- 2013 Invited Keynote Speaker, 2013 Vancouver Autophagy Symposium, Vancouver, BC, Canada
- 2013 Invited Seminar, Department of Cell and Developmental Biology, Oregon Health & Sciences University, Portland, OR.
- 2013 Invited Seminar, UCSF MSTP Grand Rounds, San Francisco, CA
- 2013 Invited Seminar, Amgen Department of Oncology, San Francisco, CA

Patents and Licenses (Months 1-12):

None.

Degrees Obtained (Months 1-12):

None.

Reagent Development (Months 1-12):

Generation of MMTV-PyMT mice on the C57B/6 strain background.
 Generation of *atg12f*, LSL-RFP mice on the C57B/6 strain background.
 Generation of *atg5f*, LSL-RFP mice on the C57B/6 strain background.
 Generation of FSP-Cre, *atg12f*, LSL-RFP mice.
 Generation of primary mammary fibroblasts and CAFs from *atg5f/f* and *atg12f/f* mice suitable for transplantation into a C57B/6 host animal.

Funding Applied For Based On Work Supported DOD Concept Scholar Award (Months 1-12):

1. Rudnick, Jenny (Post-doc): ACS Post-doctoral Fellowship
 Status: AWARDED (7/1/2013 - 6/30/2016)
 Was terminated early due to the award of BC123092.
2. Rudnick, Jenny (Post-doc): BC123093 (DOD BCRP Post-doctoral Fellowship)
 Status: AWARDED (9/15/2013-9/14/2016)
3. Debnath, Jayanta (PI): UCSF Breast Oncology Program Developmental Project
 Status: AWARDED (4/1/2013 - 3/31/2013)
4. Debnath, Jayanta (PI): Samuel Waxman Cancer Research Foundation Award
 Status: AWARDED (7/1/2013-6/30/2015)

Employment and Research Opportunities:

None.

V. CONCLUSION:

Breast cancer is a heterogeneous, multi-factorial disease whose etiology relies upon micro-environmental changes that promote tumor growth and progression. Such changes typically involve the appearance of activated fibroblasts (referred to as “cancer associated fibroblasts”, or CAFs), the recruitment of various immune cells, and enhanced type I collagen deposition, termed desmoplasia [17]. Within the last decade, a plethora of evidence demonstrates the importance of this inflammatory and desmoplastic stromal response to the initiation and progression of breast cancer [3]. Overall, the cell biological mechanisms controlling the pro-tumor functions of stromal fibroblasts, as well as CAFs remain poorly understood. This proposal seeks to dissect how autophagy, a tightly regulated lysosomal degradation process, in stromal fibroblasts directs their tumor promoting properties, and how autophagy inhibition in fibroblasts impacts their secretion of specific factors that govern aggressive behavior of breast cancer cells.

During the first year of this proposal, we have optimized the conditions necessary to isolate both normal mammary gland fibroblasts as well as PyMT-tumor derived cancer associated fibroblasts. Furthermore, we have successfully transplanted these fibroblasts into the mammary fat pad of syngeneic recipient mice in combination with polyoma middle T (PyMT) breast cancer cells. Our pilot studies indicate that PyMT tumor growth is severely impaired in the presence of autophagy deficient, but not autophagy-competent fibroblasts, providing support for our hypothesis that autophagy in stromal fibroblasts is essential for breast cancer progression. In addition, we have uncovered that autophagy-deficient fibroblasts exhibit reduced secretion of multiple pro-inflammatory cytokines in vitro as well as exhibit reduced contractility in type I collagen in vitro, suggesting that stromal autophagy supports the production of inflammatory cytokines and ECM remodeling crucial for the desmoplastic response. In the upcoming year, we will validate these exciting results as well as determine whether and how changes in cytokine secretion and ECM remodeling impact desmoplasia and breast cancer progression.

Impact: If our hypothesis is correct, this work will broaden our understanding of the precise consequences of autophagy inhibition in the stromal fibroblasts in breast cancer. Currently, there is immense interest in inhibiting autophagy to treat cancer[18]. The anti-malarial hydroxychloroquine (HCQ) is being rapidly translated into the clinical setting as an autophagy inhibitor, largely due to its long history of use in humans and its well-known toxicity profiles. Multiple clinical trials utilizing HCQ in combination with standard cancer therapies are under evaluation, including their use in breast cancer[18,19]. Despite this enthusiasm for manipulating autophagy against breast cancer, it remains largely unknown how autophagy inhibition alters the behavior of stromal cell types, and how this ultimately impacts the growth and survival of breast tumor cells. Our studies will provide essential information on whether autophagy inhibitors used clinically to treat breast cancer harbor stromal side effects that either work in unison or antagonistically with their effects on tumor cells. Finally, although we focus here on autophagy in fibroblasts, we recognize that tumor stroma is comprised of heterogeneous elements and anticipate that these studies will ultimately lay the foundation, both technical and biological, to interrogate the broader consequences of autophagy in diverse stromal constituents (e.g., endothelium, immune cells). Hence, these studies will impact how and where we should employ autophagy modulators in the therapeutic intervention against breast cancer.

VI. REFERENCES:

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VII. APPENDIX:

Curriculum Vitae of Jayanta Debnath